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Induction of SOCS-3 is insufficient to confer IRS-1 protein degradation in 3T3-L1 adipocytes ☆

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Abstract

Insulin receptor substrate (IRS)-1 is a key protein in insulin signaling. Several studies have shown that the expression of IRS-1 can be modulated by protein degradation via the proteasome and the degradation of IRS-1 can be related to insulin-resistant states. The degradation of IRS-1 has been shown to be induced by SOCS-1 and SOCS-3 via the ubiquitin pathway. The goal of our study was to determine if the induction of SOCS-3 correlated with increased IRS-1 degradation in cultured 3T3-L1 adipocytes. Interestingly, our studies have shown that there is little correlation between the induction in SOCS-3 expression and the degradation of IRS-1 in mature 3T3-L1 adipocytes. Our results clearly demonstrate that treatment with leukemia inhibitory factor (LIF) or cardiotrophin (CT)-1 strongly induces the expression of SOCS-3 in mature 3T3-L1 adipocytes, but does not affect the degradation of IRS-1. On the contrary, tumor necrosis factor (TNF) α and insulin, which very weakly induce SOCS-3 expression, have profound effects on IRS-1 degradation. In summary, our results indicate that the expression of SOCS-3 does not correlate with the degradation of IRS-1 proteins in fat cells.

Keywords: Adipocyte; IRS-1; SOCS-3; STAT3; Degradation

Insulin receptor substrates (IRS proteins) are a family of proteins that function as mediators in insulin signaling pathways. IRS proteins are phosphorylated by insulin receptors after the binding of insulin, and phosphorylated IRS proteins recruit various Src homology 2 (SH2) proteins, including phosphatidylinositol 3-kinase (PI3K), Grb2, and SHP2, Fyn, Nyk (reviewed in [1–3]). Following the activation of these proteins, multiple signaling pathways are induced and cellular responses include changes in glucose uptake, glycogen synthesis, mitogenesis, or gene expression [4]. A variety of studies have demonstrated a role for IRS-1 in somatic growth, insulin action, and glucose metabolism and insulin resistance [5]. The cellular levels of IRS-1 are affected by many effectors including growth factors and cytokines. In the last several years, many independent studies have revealed that the loss of IRS-1 protein levels is due to enhanced degradation by the ubiquitin proteasome system [6–8]. PI3K has been shown to play a role in IRS-1 degradation [9,10] and more recent studies suggest that b-arrestin affects IRS-1 degradation and insulin resistance [11]. Also, two members of the SOCS family of proteins, SOCS-1 and 3, have been shown to target IRS-1 for ubiquitin mediated degradation [12]. This observation is supported by an additional study which demonstrated that adenoviral mediated expression of SOCS-3 in the liver induces insulin resistance [13]. Hence, several studies indicate that IRS-1 ubiquitin mediated degradation can be conferred by an induction in SOCS-3 expression.

SOCS proteins comprise a family of suppressors of cytokine signaling. Each of the family members has an SH2 domain and a conserved C-terminal SOCS box [14]. The expression of CIS SOCS-1, SOCS-2, and SOCS-3 is induced by various cytokines and overexpression studies in various cell lines have demonstrated an inhibitory role of these proteins. One member of the SOCS family has been reported to act as a negative regulator of insulin

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signaling pathways [15]. Also, in adipose tissue of obese mice, the treatment of TNFα induces SOCS-3 expression [16]. It was suggested that SOCS-3 inhibits insulin signaling pathway through the inhibition of the IRS-1 tyrosine phosphorylation and the association of IRS-1 with PI3K subunit P85 [16], and through proteasome-mediated IRS-1 degradation [12]. However, whether SOCS-3 expression is sufficient to induce IRS-1 degradation in 3T3-L1 adipocytes has not been established. In our studies, we have shown that both LIF and CT-1 strongly induce SOCS-3 expression in 3T3-L1 adipocytes, while TNFα and insulin have moderate effect or almost no effect on SOCS-3 levels. On the contrary, the levels of IRS-1 protein in LIF and CT-1 treated cell groups are comparable to those of untreated adipocytes, whereas IRS-1 levels in TNFα and insulin treated cells are actively degraded. In summary, we have clearly shown that the induction of SOCS-3 does not correlate with the degradation of IRS-1 proteins in 3T3-L1 adipocytes. These studies indicate that the SOCS-3 expression alone is insufficient to confer IRS-1 degradation in cultured fat cells.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies. Bovine and fetal bovine (FBS) sera were purchased from Sigma and Invitrogen, respectively. Porcine growth hormone, insulin, and cycloheximide were purchased from Sigma. Human recombinant CT-1 was purchased from Calbiochem. Mouse recombinant LIF was purchased from Chemicon International. The highly phospho-specific polyclonal antibodies for STAT3 (Y^{705}) and STAT5 (Y^{694}) were IgGs purchased from BD Transduction Laboratories and Upstate Biotechnology, Active ERK antibody was a rabbit polyclonal IgG purchased from Cell Signaling Technology. ERK1 was a rabbit polyclonal IgGs purchased from Santa Cruz. IRS polyclonal antibody was purchased from Santa Cruz. HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch. Enhanced chemiluminescence (ECL) kit was purchased from Pierce. Nitrocellulose and Zeta Probe-GT membranes were purchased from Bio-Rad.

Cell culture. Murine 3T3-L1 preadipocytes were plated and grown to 2 days post confluence in DMEM with 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin. After 48 h, this medium was replaced with DMEM supplemented with 10% FBS, and cells were maintained in this medium until utilized for experimentation.

Preparation of whole cell extracts. Monolayers of 3T3-L1 preadipocytes or adipocytes were rinsed with phosphate-buffered saline (PBS) and then harvested in a non-denaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% Igepal CA-630 (Nonidet P-40), 1 μM PMSF, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Samples were extracted for 30 min on ice and centrifuged at 13,000 rpm at 4 °C for 10 min. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit (Pierce) according to the manufacturer's instructions.

Gel electrophoresis and Western blot analysis. Proteins were separated in 7.5% polyacrylamide (acrylamide from National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli [17] and transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% fat-free milk for 1 h at room temperature. Results were visualized with HRP-conjugated secondary antibodies and enhanced chemiluminescence.

RNA analysis. Total RNA was isolated from cell monolayers with Trizol according to manufacturer's instructions with minor modifications. For Northern blot analysis, 20 μg of total RNA was denatured in formamide and electrophoresed through a formaldehyde/agarose gel. The RNA was transferred to Zeta Probe-GT, cross-linked, hybridized, and washed as previously described [18]. SOCS-3 cDNA was labeled by random priming using the Klenow fragment and [α ^{32}P]dATP.

Results

SOCS-3 expression is significantly induced by LIF and CT-1, while TNFα and insulin have distinct effects on the induction of SOCS-3 expression

Mature 3T3-L1 adipocytes were exposed to TNFα, LIF, GH, CT-1, or insulin. Total RNA and whole cell extracts were isolated at various times after the treatments. The results in Fig. 1A demonstrate that SOCS-3 mRNA is expressed at very low levels in untreated adipocytes. However, a 1-h treatment with LIF, CT-1 or growth hormone (GH) results in a significant induction of SOCS-3 mRNA. In addition, a 3 h TNFα treatment results in an induction of SOCS-3 mRNA. Only a small induction of SOCS-3 mRNA was observed following a 1 or 3 h insulin treatment.

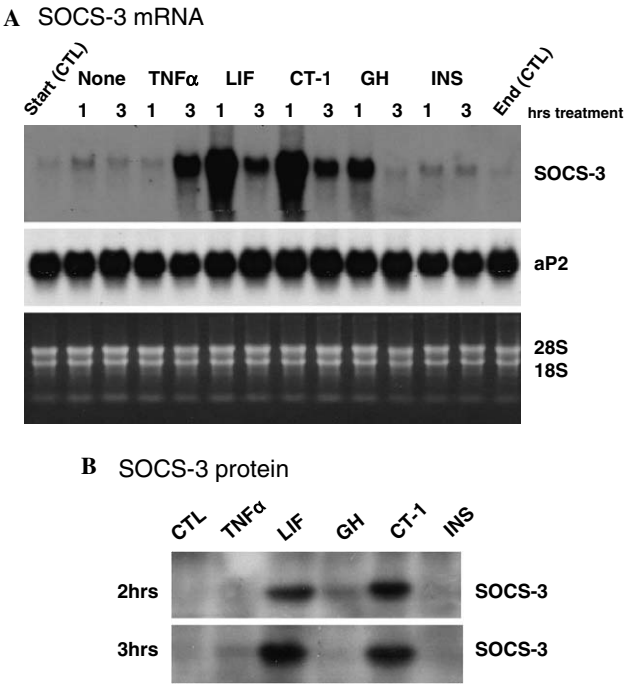

Fig. 1. The expression of SOCS-3 is highly induced by LIF and CT-1, but not by TNFα and insulin in 3T3-L1 adipocytes. Whole cell extracts and total RNA were prepared from fully differentiated serum-deprived 3T3-L1 adipocytes following a 15-min treatment of TNFα (1 nM), LIF (0.2 nM), GH (125 ng/mL), CT-1 (0.2 nM), or insulin (50 nM) for the times indicated in the figures. (A) Fifteen micrograms of total RNA was electrophoresed, transferred to nylon, and subjected to Northern blot analysis to examine SOCS-3 and aP2 mRNA accumulation. (B) One hundred micrograms of each extract was separated by SDS–PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Each of these figures represents an experiment independently performed three times.

The levels of aP2 RNA were not changed by the hormone/cytokine treatments. The levels of 28S and 18S rRNA are shown to indicate even loading of total RNA. An analysis of SOCS-3 protein levels in this experiment revealed that LIF and CT-1 highly induce the expression of this protein at both 2 and 3 h following treatment. However, there was little induction of SOCS-3 protein following TNFα, GH, or insulin treatment. These observations are consistent with the Northern blot analysis which demonstrates that GH did not induce SOCS-3 mRNA to the same extent as CT-1 and LIF.

TNFα and insulin induce IRS-1 degradation, but potent SOCS-3 activators do not induce IRS-1 degradation

To examine the efficacy of each hormone and cytokine studied, the cells were stimulated for 15 min and the activation of MAPK (ERKs 1 and 2) and STATs was examined using phospho-specific antibodies. As shown in Fig. 2A, all of the treatments resulted in the phosphorylation of MAPK. Only the untreated (−) cells had minimal levels of active MAPK which was not due to alterations in ERK1 expression. These results are consistent with numerous published studies. The results in Fig. 2A also demonstrate that LIF, CT-1, and GH resulted in the phosphorylation of STATs 3 and 5 under conditions where STAT expression did not change (data not shown).

Cells from this experiment were also used to examine IRS-1 degradation. Following a 2-h stimulation with the hormones/cytokine, the adipocytes were treated with cycloheximide to inhibit protein synthesis. We waited for 2 h after growth factor stimulation prior to adding the cycloheximide (CH) to allow for the induction in SOCS-3 protein expression which requires transcription and translation. Following the addition of the cycloheximide, whole cell extracts were isolated at various times to examine the decay of IRS-1 proteins. The results in Fig. 2B clearly demonstrate that IRS-1 is a very stable protein. Following a 10-h treatment of cycloheximide, the control cells or those treated with LIF, CT-1, or GH did not have any loss of IRS-1 protein. However, cells that were treated with either insulin or TNFα had a significant increase in decay of IRS-1 protein.

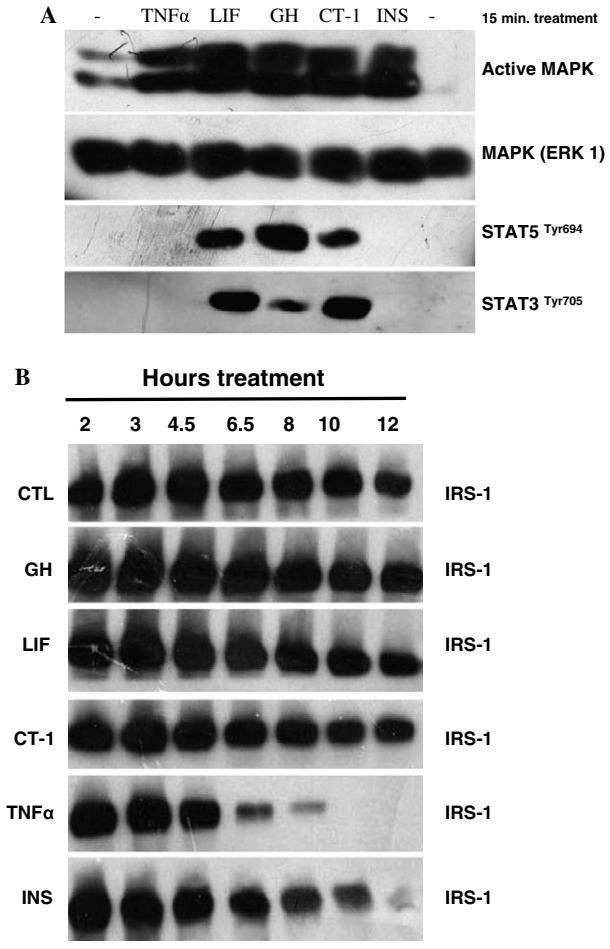

Fig. 2. The degradation of IRS-1 is induced by TNFα and insulin, but not by potent inducers of SOCS-3 expression in 3T3-L1 adipocytes. (A) Whole cell extracts were isolated from serum deprived mature 3T3-L1 adipocytes that were treated with TNFα, LIF, GH, CT-1, or insulin at the doses indicated in the previous figures. One hundred micrograms of each extract was separated by SDS–PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. (B) Mature 3T3-L1 adipocytes were maintained overnight in 2% FBS and DMEM, and were treated the following day with TNFα, LIF, GH, CT-1, or insulin as previously described. Two hours after growth factor treatment, the cells were treated with 50 μM cycloheximide and were harvested at the indicated time points after growth factor stimulation. One hundred micrograms of each extract was separated by SDS–PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Each of these figures represents an experiment independently performed three times.

Discussion

High expression levels of SOCS-3 have been shown to be controlled by activation of STAT3 in cutaneous T cell lymphoma cells [19]. Independent studies on mouse embryonic fibroblasts also revealed the interferon gamma induced SOCS-3 expression is mediated by STAT3 [20]. In our studies, we also observed that SOCS-3 expression was tightly correlated with STAT3 activation in 3T3-L1 adipocytes. Two gp130 cytokines, LIF and CT-1, strongly induced STAT3 phosphorylation, and correlated with a substantial induction of SOCS-3 levels. Whereas, insulin and TNFα, which do not induce STAT3 tyrosine phosphorylation, had minimal effects on SOCS-3 levels under physiological conditions (Fig. 1B).

A decrease of IRS-1 protein levels has been observed in conditions of TNFα and insulin induced insulin resistance in vitro and in vivo [21–23]. Recent studies indicate that SOCS-3 plays a role in IRS-1 degradation and insulin resistance. There is evidence to suggest that the expression of SOCS-3 plays a negative role in insulin signaling by inhibiting IRS-1 tyrosine phosphorylation and its association with PI3K [16], and by proteasome-mediated IRS-1 degradation [12]. However, our studies have shown that two physiological stimulators of SOCS-3 do not modulate the turnover of IRS-1 proteins in adipocytes (Fig. 2B). Howev-

er, we did observe the ability of both TNFα and insulin to substantially increase the turnover of IRS-1 proteins that was not accompanied by a substantial induction in SOCS-3 expression.

The results of our study conflict with reports which suggest that SOCS-3 induces IRS-1 degradation in transfected HEK293 cells [12]. A possible explanation for these observations is that the ectopic expression of SOCS-3 in transfected HEK293 cells is not physiological and, thus, induces IRS-1 protein degradation in a non-specific manner. The levels of SOCS-3 induced in cultured 3T3-L1 adipocytes by cytokine treatments appear to be much less than the levels of SOCS-3 expression that have been achieved in transfected cells. Although, our results indicate that SOCS-3 is not involved in IRS-1 degradation under the conditions investigated, we cannot rule out the involvement of other SOCS proteins.

In summary, our studies have shown that the IRS-1 degradation in 3T3-L1 adipocytes is induced by insulin and TNFα treatment, while the strong induction of SOCS-3 by LIF and CT-1 had no affect on IRS-1 degradation. Overall, our studies suggest that the induction of SOCS-3 is insufficient to induce IRS-1 degradation in 3T3-L1 adipocytes.

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